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(54) Title: METHODS FOR QUANTIFYING NUCLEIC ACID MOLECULES

(57) Abstract

The invention involves an assay for quantifying the number of copies of a gene, or the level of its expression, in a biological sample. The method involves using an oligonucleotide molecule which will hybridize to both the target nucleic acid molecule and at least one additional nucleic acid molecule in the sample. The competition between the target and the additional molecule or molecules results in a pattern of expression which permits quantification of the target in the sample. The method can also be used to follow infections, or the development of changes in an infectious agent.

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METHODS FOR QUANTIFYING NUCLEIC ACID MOLECULES

RELATED APPLICATION

This application is a continuation of part of Serial No. 08/907,129, filed August 6, 1997, incorporated by reference.

FIELD OF THE INVENTION

This invention relates to nucleic acid hybridization assays which are useful in quantifying a target nucleic acid molecule of interest. More particularly, it relates to the development of hybridization assays, such as PCR assays which are useful to quantify these targets. In particular, methods for quantifying nucleic acids associated with disorders, such as viral infections (e.g., human cytomegalovirus) or pathological conditions (e.g., prostate cancer) are described; however, the methodologies used for quantifying these mRNAs are useful in any hybridization assay context. Also a part of the invention are the specific primers developed for use herein. The assay, as is explained, is based upon the relationship of amplification products resulting from the amplification. The method can be used, for example, in following the success or failure of a therapeutic regime.

BACKGROUND AND PRIOR ART

The ability to diagnose and to monitor diseases, infections, etc., had been facilitated tremendously by developments in technologies related to DNA. As the nucleotide sequences of specific pathogens are determined, it is becoming much easier to determine the presence or amount of these pathogens in a particular sample, such as a body fluid sample, a cell sample, drinking water, and so forth.

Similarly, the techniques developed in this area have also facilitated, and continue to facilitate, the ability to identify genetic disorders, such as sickle cell anemia, by determining whether specific genetic mutations are present. Further, the ability to quantify the degree of expression of a particular gene, be it an abnormal gene or a normal one that is expressed at an inappropriate level or in an inappropriate tissue, facilitates diagnosis, the monitoring of diseases, and so forth.

One very powerful example of this type of technology is the area known as nucleic acid amplification. The amount of problematic DNA in a given sample, even if disproportionately large relative to the normal amount in a sample, is vanishingly small. Amplification techniques all employ the same general set of principles. These include the use of short nucleic acid molecules, or oligonucleotides ("oligos"), which are complementary to a nucleic acid molecule (the "target" or "template") to be assayed. These oligos hybridize to the target, as is elaborated upon infra, where they are acted on by DNA polymerases. These enzymes extend the oligos by adding nucleotide bases, one at a time, to the oligos. The extended oligos may then act as templates themselves. Repeated hybridization and extension achieved using cyclical reaction conditions thus results in an exponential increase of the target length. The products can be separated from the target, and serve as targets themselves for further amplification. Using these methodologies one secures, over time, sufficient copies of the target such that it is much easier to determine its presence.

The ability to detect targeted nucleic acid molecules is of great interest and importance in determining infection by pathogens. While the pathogen may be any microorganism, the case of viruses is especially germane, and is discussed herein. All of the comments presented here, however, are essentially parallel to the determination of any microorganism.

Viruses are characterized by particular nucleotide

sequences which are unique. Many serious conditions, such as hepatitis C virus infection ("HCV"), human immunodeficiency virus infection ("HIV"), human papilloma virus infection ("HPV"), and human cytomegalovirus infection ("HCMV"), are caused by these pathogens.

One of the problems with diagnosing viral infections is that they may not manifest themselves for some time following infection. For example, in the case of HCMV associated with organ transplantation, clinically observable infection may not become manifest for 1-4 months. See, e.g., Rubin, et al, Transplant. Proc. 23:2068-2074 (1991). The infection frequently leads to life threatening complications, involving the lungs, (interstitial pneumonitis). This condition is fatal in up to 48% of cases involving renal transplant. See Peterson, et al., Medicine 59:283-291 (1980). It is also common knowledge that HIV infection may not become recognized until years after the actual infection. Conversely, most infections of, e.g., HCMV, are clinically insignificant because while infection per se may be common, the infections are only significant when the level of infection is high.

Where antiviral drugs are available, their efficacy is frequently increased if administered early in the infection. Hence, the need for prompt diagnosis is clear. Many standard techniques, such as cell culture, require extended periods of time, or are not very sensitive. See, e.g., Gleaves, et al., J. Clin Microbiol 21:217-221 (1985). Serological diagnosis, which is not uncommon, does not reflect current infection, due to the lag in antibody titer increase. As such, DNA amplification assays have become more and more prevalent in the area of diagnosis of such pathogens.

Other pathological conditions are characterized by expression of genes at levels, which differ from normal levels, or by expression of genes which are not normally expressed. There are many examples of these conditions which are known to the artisan, and they need not be set forth here. Exemplified in this application is prostate specific antigen ("PSA"), which is linked to prostate cancer.

An important problem associated with cancer is to determine whether or not the primary tumor has metastasized. This can be done by assaying for a molecule associated with the tumor at a second site. In the case of prostate cancer a possible assay is to determine the presence of PSA in the circulation as an indication of probable bone marrow metastasis. See e.g., Moreno et al, Cancer Res. 52: 6110-6112 (1992) and Israeli et al, Cancer Res. 54: 6306-6310 (1994). This is done by detecting the presence of the mRNA of this molecule. Since very low levels of the mRNA may be present in the circulation of normal individuals, the quantification of the PSA mRNA is relevant.

One type of DNA amplification assay is the polymerase chain reaction, or "PCR". This method, which is widely known, utilizes oligonucleotide primers, as described supra, in combination with heat stable polymerases, such as "Taq" DNA polymerase, or "Pfu" polymerase.

There is a basic difference in qualitative (yes/no) hybridization assays, and quantitative assays, which determine the starting amount of target nucleic acid. It is the latter aspect which is discussed herein.

DNA quantification via PCR falls into one of two categories, i.e., semi-quantitative assays, and competitive assays. Each is discussed herein.

Semi-quantitative assays can be divided into three groups: end point dilution prior to PCR (Kulski, et al. J. Virol. Meth. 49:195-208 (1994); co-amplification of target and cellular DNA (Kellogg, et al., Anal. Biochem. 189:202-208 (1990)); and a comparison assay, based upon comparing a sample PCR product, to a product obtained via serial dilution of external standards (Terry, et al., Arch. Virol 128: 123-133 (1993). These assays provide relative, rather than absolute results, since the efficiency of amplification is not controlled.

The majority of quantitative PCR assays are competitive in nature. In these assays, target DNA, and one internal standard, are co-amplified. The standard is modified from the target, by size, presence or absence of a restriction site.

The competitive PCR methods known to the art do permit control of the efficiency of amplification. The problem with these systems is that internal standards must be constructed and added to the reactions. This must be carried out several times, with co-amplification of different amounts of the internal standard and control. As a result, the ability to carry out assays on significant numbers of samples is diminished.

Further, very accurate determination of the amount of biological materials being assayed for are required for meaningful quantitation.

Applicants have discovered a method that eliminates the need for these arduous, time consuming steps which nonetheless permits the artisan to quantify the number of copies of a gene of interest or to assay for expression of a gene of interest.

In standard approaches to quantifying the number of copies of a gene of interest, or the level of its expression, a set of conditions as stringent as is possible is developed. Stringent conditions generally involve the design and use of an oligonucleotide probe/primer which is as specific for the target as possible. In other words, one looks for an oligonucleotide which hybridizes only to target, and not other partners. Then, the specific assay is run at conditions which maximize the hybridization to only the target. It is well known that, as stringency decreases, the amount of cross-hybridization increases. It has now been found, however, that one can actually quantify a target nucleic acid molecule in a sample by reducing stringency. In so doing, there is no need to add internal standards to the reaction. To the contrary, one adjusts the assay conditions until a desired number of bands results. Since, under the reduced stringency conditions being used, one expects competition, one can quantify the target molecule via comparing the degree of amplification of the target to the expression of other competing molecules. Generally, this is done by comparing the intensity of amplified bands in a gel.

The features of this invention will become clear in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1E, inclusive, show the effect of varying the temperature of a PCR assay, on sets of primers.

Figure 2 depicts results obtained when HCMV positive samples were assayed with the optimal pair of primers found following the work shown in figures 1A-1E.

Figure 3 presents results obtained using varying amounts of HCMV DNA.

Figure 4 is a standard curve developed from the data of figure 3.

Figure 5 presents data obtained in experiments designed to follow a therapeutic regime.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention will be described generally and then elaborated upon in the examples which follow.

When quantifying the number of copies, or expression of a gene with a known nucleotide sequence, one can easily prepare oligonucleotide probes or primers of a desired length, using well known rules of base pairing. For example, an oligonucleotide of formula $(A)_{10}$ has, as its complement $(T)_{10}$, while $(C)_{10}$ has as its complement $(G)_{10}$. Under the usual set of high stringency conditions, the difficulty is developing a probe or primer which is so specific for the target that there is no competition or as little competition as possible. Developing such oligonucleotide becomes a highly unpredictable, very empirical enterprise.

In the invention, one simply uses a probe/primer of desired length, generally from 10-50 nucleotides in length, which will hybridize to a target. The hybridization need not result from 100% complementarity; indeed, anywhere from about 50 to up to 100% complementarity is feasible.

The oligonucleotide will hybridize to the target, but if the conditions of stringency are reduced, it will also hybridize to other, non-target sequences. This may seem undesirable, but it is key to the invention.

Following the carrying out of the non-specific amplification assay, one studies the pattern of amplification via, e.g., separating amplification products on a gel, by means of the size of the product. Anywhere from one extra, to dozens of extra bands may be produced.

The key to the invention is that one modifies the conditions until the number of extra bands is reasonably small, i.e., anywhere from 1 to 10, preferably 1 to 5, and most preferably one other band. Since the non-target sequences will compete for the probe, study of the banding pattern resulting from the new condition permits the artisan to ascertain the number of copies of target especially if working with a set of predetermined control values, which have also been determined in the manner suggested herein.

How this and other features of the invention are realized will now be set forth.

EXAMPLE 1

This example demonstrates how the low stringency hybridization and amplification methodology functions, in the context of PCR.

A PCR assay has been described by Shibata, et al. J. Infect Dis 158: 1158-1192 (1988), which is incorporated by reference. The assay determines human cytomegalovirus ("HCMV"), by amplification of a 136 base pair sequence of the late antigen gp64, which is common to all known HCMV strains.

Shibata et al used, as primers:

5'-CCGCAACCTG GTGCCCATGG-3'

and

5'-CGTTTGGGTT GCGCAGCGGG-3'

(SEQ ID NOS: 1 and 2, respectively).

These primers will be referred to as LA1 and LA2 herein.

A set of modified versions of LA1 were then prepared as follows:

LA1m1: identical to LA1 except the base at the 3'-end base is C rather than G.

LA1m2: identical to LA1, except the penultimate 3'-end base is C rather than G.

LA1m3: identical to LA1 except the base 5 units from the 3'-end is G rather than C.

LA1m4: identical to LA1 except the base 7 units from the 3'-end is G rather than C.

Each of LA1, LA1m1, LA1m2, LA1m3, and LA1m4 were then combined, together with LA2, to carry out PCR using DNA extracted from peripheral blood lymphocytes of HCMV positive patients. For a 50 ul reaction, 5-100 ng of DNA were used. Standard methods were used to extract the DNA. In these assays, 12.5pmols of each primer were combined with Tris-HCl (10mM) (pH 8.3) KCl (50mM), $MgCl_2$ (1.5mM), and 2 units of Taq DNA polymerase, together with distilled water, to bring the reaction volume to 50ul. To carry out the PCR, a first cycle was carried out at 94°C, for 5 minutes (denaturation) followed by annealing, for 1 minute (at varying temperatures: see below), followed by 1 minute of extension at 72°C. This was followed by 32 cycles (94°C, 30 seconds; varying temperature, 1 minute, 72°C, 1 minute), and a single, ending cycle which paralleled cycles 2-33, except the extension reaction was carried out for 8 minutes.

As indicated, supra, annealing was carried out at varying temperatures. These were 72°C (high stringency), 68°C and 62°C (both of which are reduced stringency). A 5ul sample of each reaction was fractionated on 6% polyacrylamide gel, and visualized by silver staining.

Figure 1A shows the results obtained when LA1 and LA2 were used at high stringency. Note the amplification of only the specific band, followed by a few, faint bands at 68°C, and multiple banding at 62°C. This experiment showed that, in this case, simply reducing the temperature of the annealing step of the assay did not result in an amplification profile that was suitable for quantifying very low amounts of the target sequence. The ideal profile as discussed supra would contain the target gene sequence and one other nonspecific product.

To obtain the necessary reduction in stringency that results in the desired amplification profile, one of the two oligos was altered and the assay repeated.

Figure 1B shows parallel results using LA1m1, and show that a number of bands are seen under reduced stringency, as well as the specific band. The banding pattern formed at 62°C is complex.

In figure 1C, the results obtained using LA1m2 and LA2 are shown, and no banding is seen at either 68°C or 72°C, while a relatively small number of bands are seen at 62°C, one of which is close to the specific band in terms of its intensity.

When LA1m3 was used together with LA2 (figure 1D), the specific band was amplified at 72°C, but amplification was less efficient than was observed with the other primers. At 68°C, a number of products were seen, one of which is close in size to the specific band.

The banding obtained when LA2 and LA1m4 were used is shown in figure 1E. The specific band is present at 72°C, with a single, strong additional band, at 68°C. This band is close to the target in size. The fact that only two bands were visualized make this an ideal system for quantifying the nucleic acid molecule of interest. Note that at 62°C, multiple banding results, leading to a reduction in the intensity of the desired banding.

This experiment thus shows how to identify the ideal primer and conditions for carrying out a reduced stringency amplification assay. In the experiments which follow, 68°C and the primer pair of LA2 and LA1m4 were used.

EXAMPLE 2

Using the protocols described in example 1, supra, a PCR assay was carried out on DNA extracted from PBLs patients who had detectable systemic HCMV infection. LA2 and LA1m4 were used as primers.

The results for twelve samples are presented in figure 2. Lane 1 presents molecular weight markers, and lanes 2-13, the samples. The patients had various degrees of infection, hence the results showed that the method of the invention is usable in detecting varying levels of the target nucleic acid molecule.

EXAMPLE 3

A quantification assay was then developed. To do this, the HCMV sequence amplified by the primers LA2 and LA1m4 was inserted into pUC18. Following the replication of the plasmid, varying levels of it were mixed with human DNA which had been extracted from normal peripheral blood lymphocytes, ranging from 10^6 copies/10^6 cells, down to 10 copies/10^6 cells. The assay was then carried out, as described supra. The assay is clinically useful because earlier observations indicate that the HCMV viral load in asymptomatic patients is fewer than 50 copies per 10^6 cells. (Urushibara, et al. Jpn. J. Transfus. Med 39:544-549 (1993)); while patients with clinically significant HCMV disease have at least 10^3 copies/10^6 cells (Gerdes, et al Transplantation 25(1):1411-1413 (1993); Gerna, et al., Scand. J. Infect. Dis. Supp 99:11-15 (1995)).

Figure 3 sets forth the results. Note the increase in banding intensity as the HCMV copy number increases. Upper and lower detection limits are 10^5 and 10 copies, per 10^6 cells; above and below these levels, one of the bands cannot be detected. The quantification range is such that it extends over reported viral loads in subjects.

Using standard methodologies with the log of the HCMV/LSP210 ratio, a graph was plotted, and is shown in figure 4 (LSP210 is the arbitrary name given to the second band). Preparing such curves is standard practice for quantification assays of this type.

EXAMPLE 4

An important feature of an amplification assay is its ability to determine viral load, regardless of the amount of DNA present in the PCR reaction. To test the assay described herein, different amounts of samples taken from human subjects positive for HCMV (10ng, 50ng, 150ng, and 200ng), were amplified together with 100ng of each HCMV standard. Following electrophoreis and silver staining, the intensities of both the human and HCMV band were evaluated. Copy number was calculated, and the results are presented in the table which follows. It will be seen that, variations of up to 20 times in total DNA quantity, did not significantly alter results.

Quantity of DNA in the reaction (TOTAL)	HCMV COPY NUMBER/10^6 CELLS
10 ng	1.5×10^3
50 ng	0.9×10^3
150 ng	1.1×10^3
200 ng	1.2×10^3

EXAMPLE 5

In order to test the interassay variability for quantifying viral copy number in the same human sample, an HCMV positive sample was assayed on different days. The table, which follows, shows that the assay exhibits good reproducibility.

Date	HCMV copy number/10^6 cells
1/11/96	1.7×10^3
6/11/96	0.8×10^3
15/11/96	1.0×10^3
20/11/96	1.0×10^3

EXAMPLE 6

The experiments set forth in this example show the efficacy of the invention in determining metastasis of prostate cancer. Early detection of metastasis is important, because the development of a therapeutic regime involves consideration of various issues which include the extent of the disease. While various diagnostic tests are available for determining the level of prostate specific antigen ("PSA" hereafter) in blood, these assays are not predictive of metastasis, or of pathological state. There are various references which show the use of PCR in detecting mRNA for PSA, but these do not permit staging of the disease. The reason for this is that as the sensitivity of the assay increases, mRNA for PSA is found in the blood of healthy individuals, which compromises the value of the test. The experiments which follow describe a methodology which avoids these problems.

The following two primers, known to the art, were used:

5'-ACA CAG GCC AGG TAT TTC AG-3'
5'-GTC CAG CGT CCA GCA CCA AG-3'

SEQ ID NOS: 3 and 4, respectively. These primers are known to amplify a 355 base pair fragment from exon 5 of the PSA gene. See Israeli et al, Cancer Res. 54: 6306-6310 (1994), incorporated by reference.

An assay was carried out, wherein 10ul of isolated cDNA was combined, in a 40$\mu\ell$ reaction volume, with Tris-HCl (10mM), KCl (50mM), $MgCl_2$ (1.8mM), mixed dNTPs (250 um), 20pmol of each primer, and 2.5U of Taq DNA polymerase. The mixture was denatured by heating to 95°C, for 5 minutes. This was followed by a one minute period to permit annealing of the primers. This annealing step took place at different temperatures, as described herein. Then, PCR was carried out for 35 cycles (one cycle: 95°C for 1 minute, a varying, annealing temperature for 1 minute, 72°C for two minutes). Each reaction was analyzed by taking 5$\mu\ell$ samples of reaction product, placing these on a 7% polyacrylamide gel, subjecting the material to electrophoresis and staining with silver.

The assays were carried out by mixing cDNA from cells of a healthy female subject (total lymphocyte cDNA), together with cDNA taken from a prostate carcinoma cell line. In the assays, cDNA from 10^6 normal lymphocytes was mixed with cDNA from 1, 10 or 100 prostate carcinoma cells. It was possible to quantify the amount of PSA cDNA using assays of the type described herein. Specifically, it was found that at 68°C, i.e., "high stringency", only a single band, i.e., the expected band for PSA, was found. On the other hand, reducing the temperature to 65°C resulted in generation of a second band, which permitted a direct comparison, and a quantification. In this example, oligonucleotide alteration was not necessary to achieve the amplification profile required. As the number of copies of the PSA cDNA was increased, the intensity of the banding, relative to the reference band increased, permitting a direct quantification thereof.

EXAMPLE 7

This example describes the application of the method described herein in following a therapeutic regime.

A subject with a diagnosed CMV infection, receiving standard antiviral therapy (Ganciclovir, and then Foscarnet) was monitored, over a period of time (180 days), using the methodology described supra. The CMV viral load determined in

accordance with the invention was plotted as a function of time at Figure 5. It can be seen that the viral load dropped drastically following the first treatment with Ganciclovir. The increase in viral load following the second treatment indicates the development of resistance, requiring a change in therapy. Following Foscarnet treatment, the same decrease is observed.

Thus, one can monitor a cause of treatment, progressing regression, or status of a particular condition, by comparing two values obtained with this method, at two points in time, wherein a difference in values is indicative of a change in status, such as regression, progression, or even stasis in the condition being monitored, as well as the development of resistance by an infectious agent.

The foregoing examples illustrate the invention, which is a method for quantifying amount of a target nucleic acid molecule in a sample. Essentially, the method involves the competition for an oligonucleotide molecule (a "probe" or a "primer"), by DNA corresponding to the target nucleic acid molecule, and one or more other DNA molecules in a given sample. "DNA corresponding to the target nucleic acid molecule" refers to transcript, i.e., the first level of expression of a given nucleic acid molecule.

It is well known that transcript of a given nucleic acid molecule may be present in one or more than one copy. In the case of infection, such as bacterial or viral infection, the number of copies of pathogenic DNA may be several orders of magnitude higher than they would be in an uninfected individual or one with a latent infection. When the amount of oligonucleotide molecule(s) is kept at a constant, more will hybridize to the nucleic acid at higher copy number than would if the number were lower. The hybridization in the case of an amplification assay, such as PCR (polymerase chain reaction), leads to higher amounts of amplification product which can then be measured in, e.g., an assay which monitors the intensity of a band of a given size on a gel.

The conditions necessary for provoking the required competition can be ascertained by, e.g., varying the working conditions from calculable stringent conditions. For example, one theoretical means of estimating the temperature at which a hybridization assay is "stringent" is to use the formula:

$$Tm = 4(G+C) + 2(A+T)$$

wherein G,C, A and T are the number of times each nucleotide base appears in a given oligonucleotide. The formula provides an appropriate high stringency temperature, in Celsius degrees. In practice, this theoretical temperature is normally 3-12°C lower than the effective, ideal high stringency temperature, i.e., the temperature at which only the specific target of interest is amplified.

Generally, one can lower this stringency by dropping the temperature at which the annealing step of the assay is carried out. As a result, more than one application product can, and does result. Most desirably, it is only one additional competing product, but the method described herein would be expected to work at, e.g., 20 additional bands or more, but is most preferably carried out at conditions which yield 10 or fewer, preferably only one, additional band.

One can produce control patterns, as indicated in the examples relating to HCMV, supra, which then permit the skilled artisan to determine copy number in a test sample.

As indicated, the method works for infection, such as HCMV infection, but also human papilloma virus ("HPV"), hepatitis C virus ("HCV"), human immuno deficiency virus ("HIV"), and so forth Bacteria, such as H.pylori, E.coli, P.aeruginosa, and other microbes, such as Saccharomyces and other yeast, other Pseudomonas, Yersinia, and so forth, can also be quantitated via this method.

Further, one can monitor the course of a therapeutic regime by employing the method of the invention before and after administration of a drug, delivery of a particular therapeutic method, and so forth. By so doing, one can determine whether a

particular therapeutic approach is working, is failing, or is having no effect. Further, one can monitor changes in an infection, such as the development of resistance, by comparing data obtained at two or more points in time.

As the examples relating to PSA show, the method can also be used to "stage" diseases. As indicated, prostate cancer changes from a localized to a metastasized condition, and this can be determined via assaying for, e.g., PSA expression at a site distant from the prostate gland.

It was indicated, supra, that one can provoke the competition utilized in the assay by lowering the temperature. It can also be provoked by changing the sequence of the given oligonucleotides from those which are 100% complementary to the target, to primers which vary from 100%. The exemplification on HCMV shows the efficacy of this approach. As indicated, the oligonucleotide primers are preferably 10 to 50 nucleotides long, most preferably about 17 to 25 nucleotides in length.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

CLAIMS

1. A method for quantifying amount of expression of a nucleic acid molecule of interest in a sample, comprising:

(i) contacting said sample with an oligonucleotide molecule which hybridizes to said nucleic acid molecule of interest, and one other nucleic acid molecule in said sample,

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule of interest and said one other nucleic acid molecule in said sample, and

(iii) comparing the amounts of hybridization determined in (ii) as a determination of amount of said nucleic acid molecule of interest in said sample.

2. The method of claim 1, wherein said method is an amplification assay.

3. A method for quantifying expression of a nucleic acid molecule of interest in a sample, comprising:

(i) contacting said sample with a pair of oligonucleotide primers which hybridize to both said nucleic acid molecule and one other additional nucleic acid molecule in said sample,

(ii) amplifying said nucleic acid molecules,

(iii) determining amount of amplification product produced in (ii), and

(iv) comparing amount of amplification product in (iii) to quantify expression of said nucleic acid molecule of interest.

4. The method of claim 3, wherein said nucleic acid molecule of interest is a nucleic acid molecule foreign to a subject from which said sample is taken.

5. The method of claim 4, wherein said nucleic acid molecule of interest is a viral nucleic acid molecule.

6. The method of claim 5, wherein said viral nucleic acid molecule is an HCMV, HPV, HCV or HIV specific molecule.

7. The method of claim 3, wherein said nucleic acid molecule is indicative of a pathological condition.

8. The method of claim 7, wherein said pathological condition is an infection.

9. The method of claim 7, wherein said pathological condition is cancer.

10. The method of claim 9, wherein said cancer is prostate cancer.

11. The method of claim 7, wherein said pathological condition is metastasized cancer.

12. The method of claim 3, comprising contacting said sample with said pair of oligonucleotide primers at low stringency conditions.

13. The method of claim 12, wherein said low stringency conditions comprise a temperature below the temperature at which said oligonucleotide primers hybridize to only said nucleic acid molecule of interest.

14. The method of claim 3, wherein said pair of oligonucleotide primers are from 10 to 50 nucleotides in length.

15. The method of claim 14, wherein said pair of oligonucleotide primers are from 17 to 25 nucleotides in length.

16. The method of claim 3, wherein said pair of oligonucleotide primers are not completely complementary said nucleic acid molecule of interest.

17. The method of Claim 6, wherein said viral nucleic acid molecule of interest is an HCMV specific molecule.

18. The method of Claim 17, comprising contacting said sample with an oligonucleotide with a nucleotide sequence consisting of SEQ ID NO: 2 and an oligonucleotide with a nucleotide sequence consisting of 5' - CCGCAACCTG GTGGCCATGG - 3'

19. The method of Claim 18, comprising amplifying said HCMV specific nucleic acid molecule via polymerase chain reaction comprising heating said sample and said oligonucleotide at an annealing temperature of 68°C.

20. A method for determining progression of a pathological condition, comprising:

(i) contacting a sample taken from a patient with said pathological condition with an oligonucleotide molecule which hybridizes to a nucleic acid molecule characteristic of said condition and to one other nucleic acid molecule in said sample,

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule characteristic of said condition, and with said one other nucleic acid molecule,

(iii) comparing amounts of hybridization determined in (ii) as a determination of said nucleic acid molecule characteristic of said pathological condition, and

(iv) comparing results from (i), (ii), and (iii) t results secured by carrying out (i), (ii), and (iii) at a previous point in time, a difference in results being indicative of a change in said pathological condition.

21. The method of claim 20, wherein said pathological condition is a viral infection.

22. The method of Claim 21, wherein said viral infection is a cytomegalovirus infection.

23. The method of Claim 20, comprising carrying out (i), (ii) and (iii) following administration of a therapeutic agent.

24. A method for determining a change in an infectious agent in a patient infected with said infectious agent, comprising:

(i) contacting a sample taken from a patient with said infectious agent with an oligonucleotide molecule which hybridizes to a nucleic acid molecule characteristic of said condition and to one other nucleic acid molecule in said sample,

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule characteristic of said condition, and to said one other nucleic acid molecule,

(iii) comparing amounts of hybridization determined in (ii) as a determination of said nucleic acid molecule characteristic of said infectious agent, and

(iv) comparing results from (i), (ii) and (iii) at a previous point in time, a difference in results being indicative of a change in said infectious agent.

25. The method of Claim 24, wherein said patient is receiving a therapeutic agent, said change in said infectious agent being development of resistance to said therapeutic agent.

26. The method of Claim 24, wherein said infectious agent is a virus.

27. The method of Claim 26, wherein said virus is cytomegalovirus.

AMENDED CLAIMS

[received by the International Bureau on 18 December 1998 (18.12.98);
original claims 1,3,16,20 and 24 amended; remaining claims unchanged (5 pages)]

1. A method for quantifying amount of expression of a nucleic acid molecule of interest in a sample, comprising:

(i) contacting said sample with an oligonucleotide molecule which hybridizes to said nucleic acid molecule of interest, and one other nucleic acid molecule in said sample, wherein said one other nucleic acid molecule is a nucleic acid molecule present in said sample prior to said contacting of said oligonucleotide molecule, and is not an added internal standard,

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule of interest and said one other nucleic acid molecule in said sample, and

(iii) comparing the amounts of hybridization determined in (ii) as a determination of amount of said nucleic acid molecule of interest in said sample, to quantify amount of said nucleic acid molecule in said sample.

2. The method of claim 1, wherein said method is an amplification assay.

3. A method for quantifying expression of nucleic acid molecule of interest in a sample, comprising:

(i) contacting said sample with a pair of oligonucleotide primers which hybridize to both said nucleic acid molecule of interest and one other additional nucleic acid molecule in said sample,

(ii) amplifying said nucleic acid molecules,

(iii) determining amount of amplification product produced in (ii), and

(iv) comparing amount of amplification product in (iii) to quantify expression of said nucleic acid molecule of interest.

4. The method of claim 3, wherein said nucleic acid molecule of interest is a nucleic acid molecule foreign to a subject from which said sample is taken.

5. The method of claim 4, wherein said nucleic acid molecule of interest is a viral nucleic acid molecule.

6. The method of claim 5, wherein said viral nucleic acid molecule is an HCMV, HPV, HCV or HIV specific molecule.

7. The method of claim 3, wherein said nucleic acid molecule is indicative of a pathological condition.

8. The method of claim 7, wherein said pathological condition is an infection.

9. The method of claim 7, wherein said pathological condition is cancer.

10. The method of claim 9, wherein said cancer is prostate cancer.

11. The method of claim 7, wherein said pathological condition is metastasized cancer.

12. The method of claim 3, comprising contacting said sample with said pair of oligonucleotide primers at low stringency conditions.

13. The method of claim 12, wherein said low stringency conditions comprise a temperature below the temperature at which said oligonucleotide primers hybridize to only said nucleic acid molecule of interest.

14. The method of claim 3, wherein said pair of oligonucleotide primers are from 10 to 50 nucleotide in length.

15. The method of claim 14, wherein said pair of oligonucleotide primers are from 17 to 25 nucleotide in length.

16. The method of claim 3, wherein said pair of oligonucleotide primers are not completely complementary to said nucleic acid molecule of interest.

17. The method of Claim 6, wherein said viral nucleic acid molecule of interest is an HCMV specific molecule.

18. The method of Claim 17, comprising contacting said sample with an oligonucleotide with a nucleotide sequence consisting of SEQ ID NO: 2 and an oligonucleotide with a nucleotide sequence consisting of 5' - CCGCAACCTG GTGGCCATGG - 3'

19. The method of Claim 18, comprising amplifying said HCMV specific nucleic acid molecule via polymerase chain reaction comprising heating said sample and said oligonucleotide at an annealing temperature of 68°C.

20. A method for determining progression of a pathological condition, comprising:

(i) contacting a sample taken from a patient with said pathological condition with an oligonucleotide molecule which hybridizes to a nucleic acid molecule characteristic of said condition and to one other nucleic acid molecule in said sample, wherein said one other nucleic acid molecule is a nucleic acid molecule present in said sample prior to said contacting of said oligonucleotide, and is not an added internal standard,

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule characteristic of said condition, and with said one other nucleic acid molecule,

(iii) comparing amounts of hybridization determined in (ii) as a determination of said nucleic acid molecule characteristic of said pathological condition, and

(iv) comparing results from (i), (ii), and (iii) to results secured by carrying out (i), (ii), and (iii) at a previous point in time, a difference in results being indicative of a change in said pathological condition.

21. The method of claim 20, wherein said pathological condition is a viral infection.

22. The method of Claim 21, wherein said viral infection is a cytomegalovirus infection.

23. The method of Claim 20, comprising carrying out (i), (ii) and (iii) following administration of a therapeutic agent.

24. A method for determining a change in an infectious agent in a patient infected with said infectious agent, comprising:

(i) contacting a sample taken from a patient with said infectious agent with an oligonucleotide molecule which hybridizes to a nucleic acid molecule characteristic of said condition and to one other nucleic acid molecule in said sample, wherein said one other nucleic acid molecule is a nucleic acid molecule present and in said sample prior to said contacting of said oligonucleotide molecule, and is not an added internal standard.

(ii) determining amount of hybridization of said oligonucleotide with said nucleic acid molecule characteristic of said condition, and to said one other nucleic acid molecule,

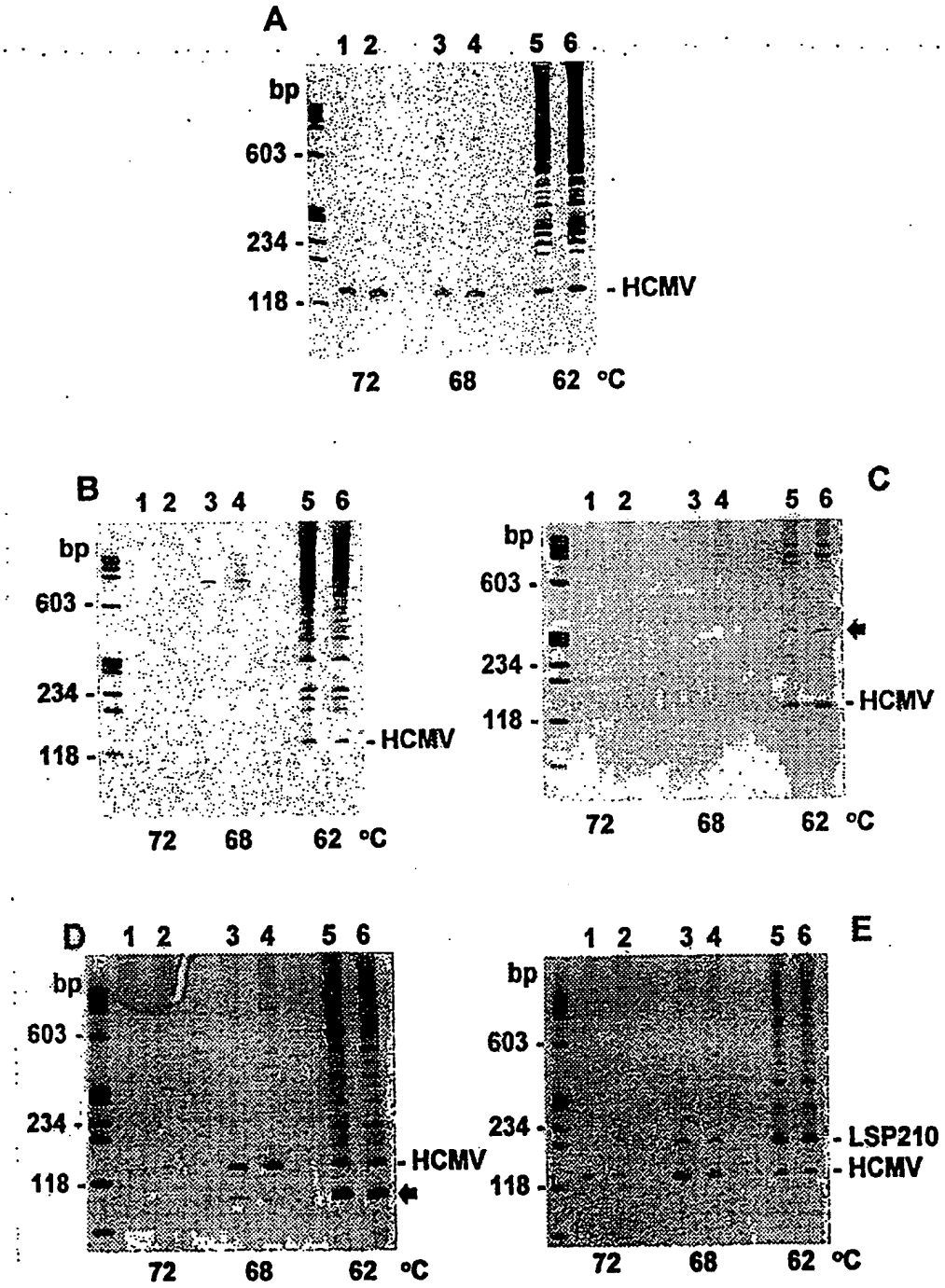
(iii) comparing amounts of hybridization determined in (ii) as a determination of said nucleic acid molecule characteristic of said infectious agent, and

(iv) comparing results from (i), (ii) and (iii) at a previous point in time, a difference in results being indicative of a change in said infectious agent.

25. The method of Claim 24, wherein said patient is receiving a therapeutic agent, said change in said infectious agent being development of resistance to said therapeutic agent.

26. The method of Claim 24, wherein said infectious agent is a virus.

27. The method of Claim 26, wherein said virus is cytomegalovirus.


A
1 2 3 4 5 6
bp
603
234
118
HCMV
72 68 62 °C
B
1 2 3 4 5 6
bp
603
234
118
HCMV
72 68 62 °C
C
1 2 3 4 5 6
bp
603
234
118
HCMV
72 68 62 °C
D
1 2 3 4 5 6
bp
603
234
118
HCMV
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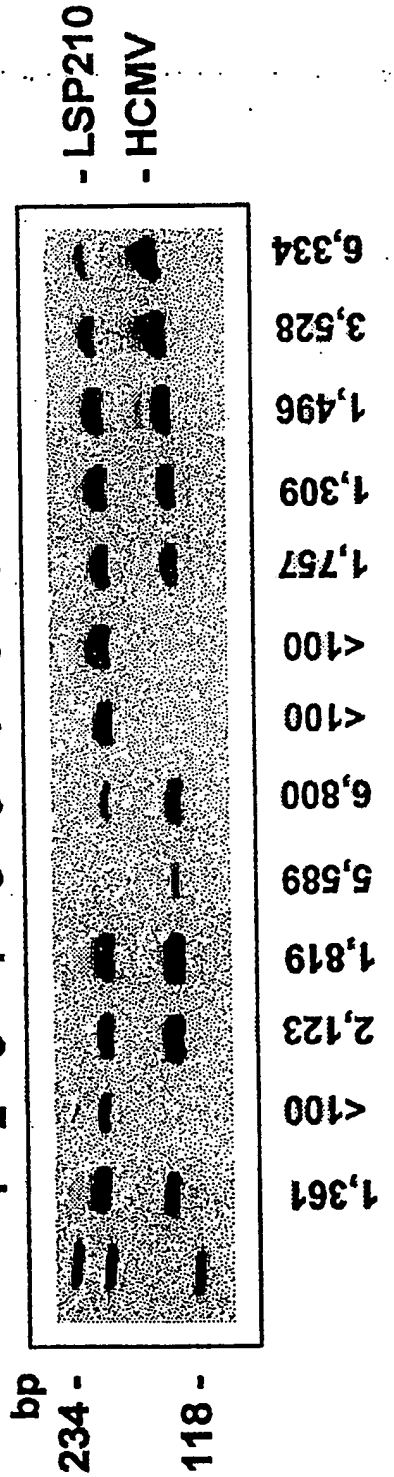
E
1 2 3 4 5 6
bp
603
234
118
LSP210
HCMV
72 68 62 °C

FIG. 1


- LSP210
- HCMV
1 2 3 4 5 6 7 8 9 10 11 12 13
1,361
<100
2,123
1,819
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<100
1,757
1,309
1,496
3,528
6,334
bp
234 -
118 -

FIG. 2

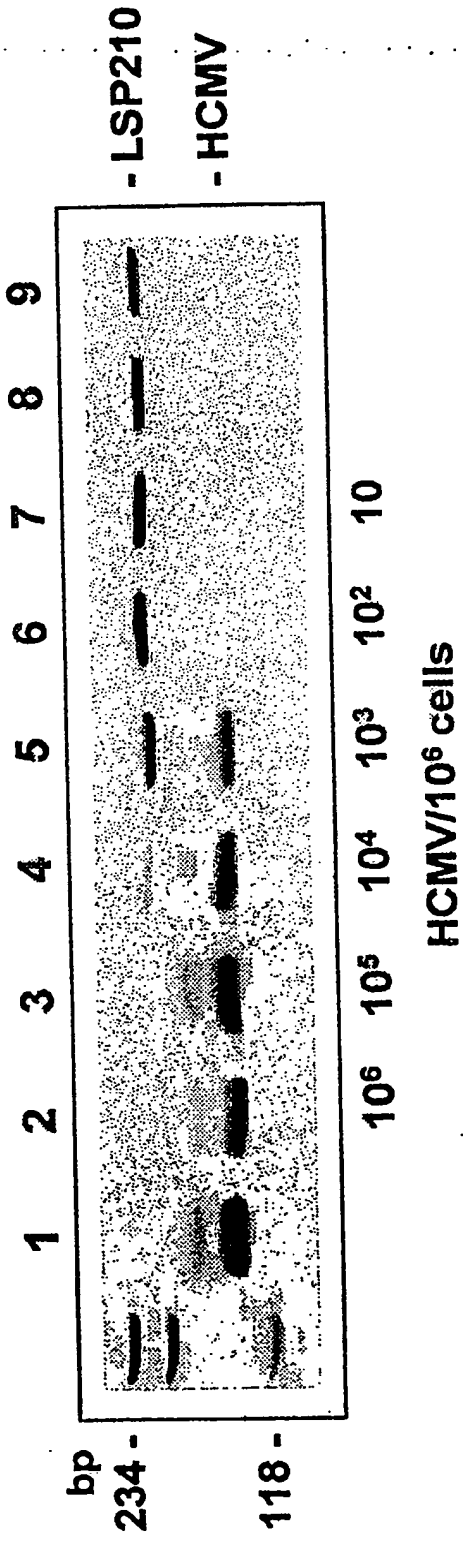
- LSP210
- HCMV
1 2 3 4 5 6 7 8 9 10
10^6 10^5 10^4 10^3 10^2
HCMV/10^6 cells
bp
234 -
118 -

FIG. 3

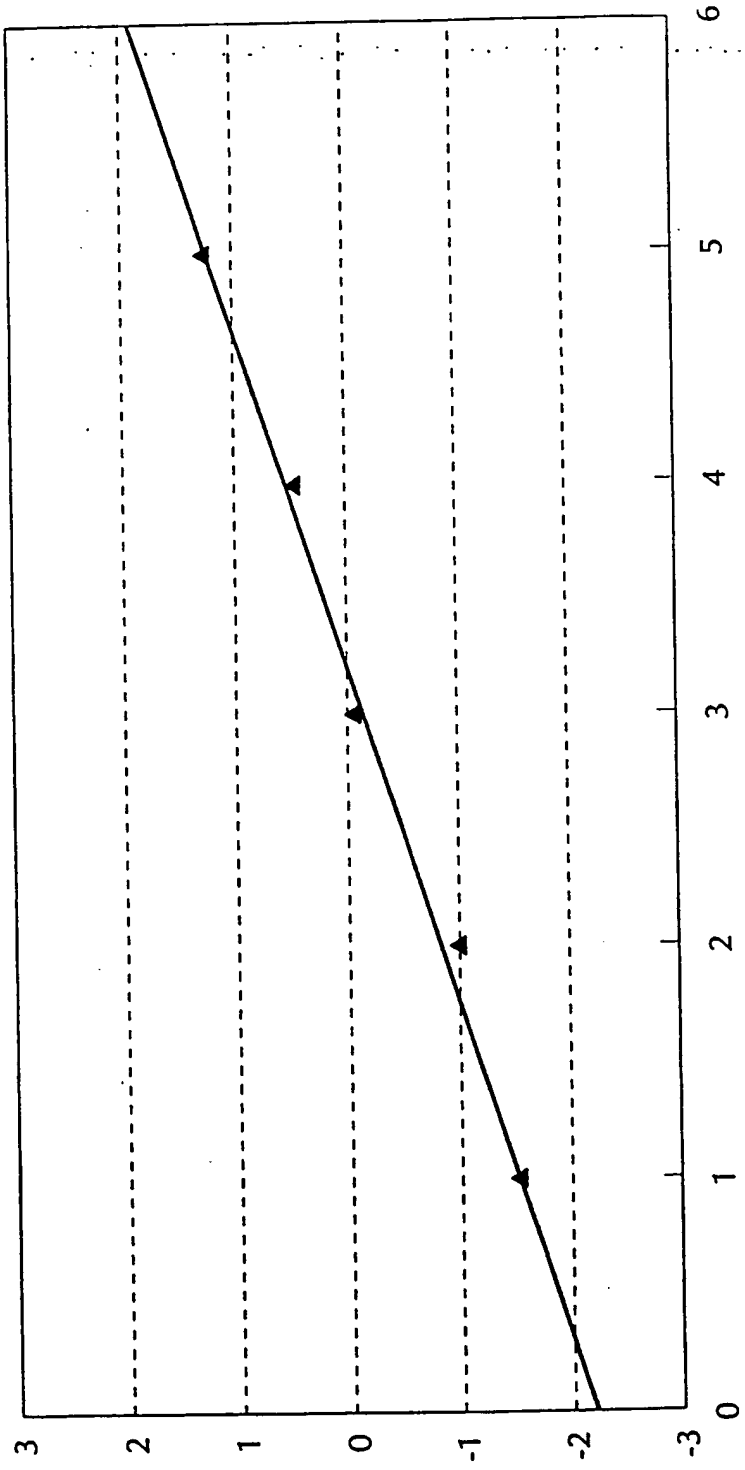

FIG. 4

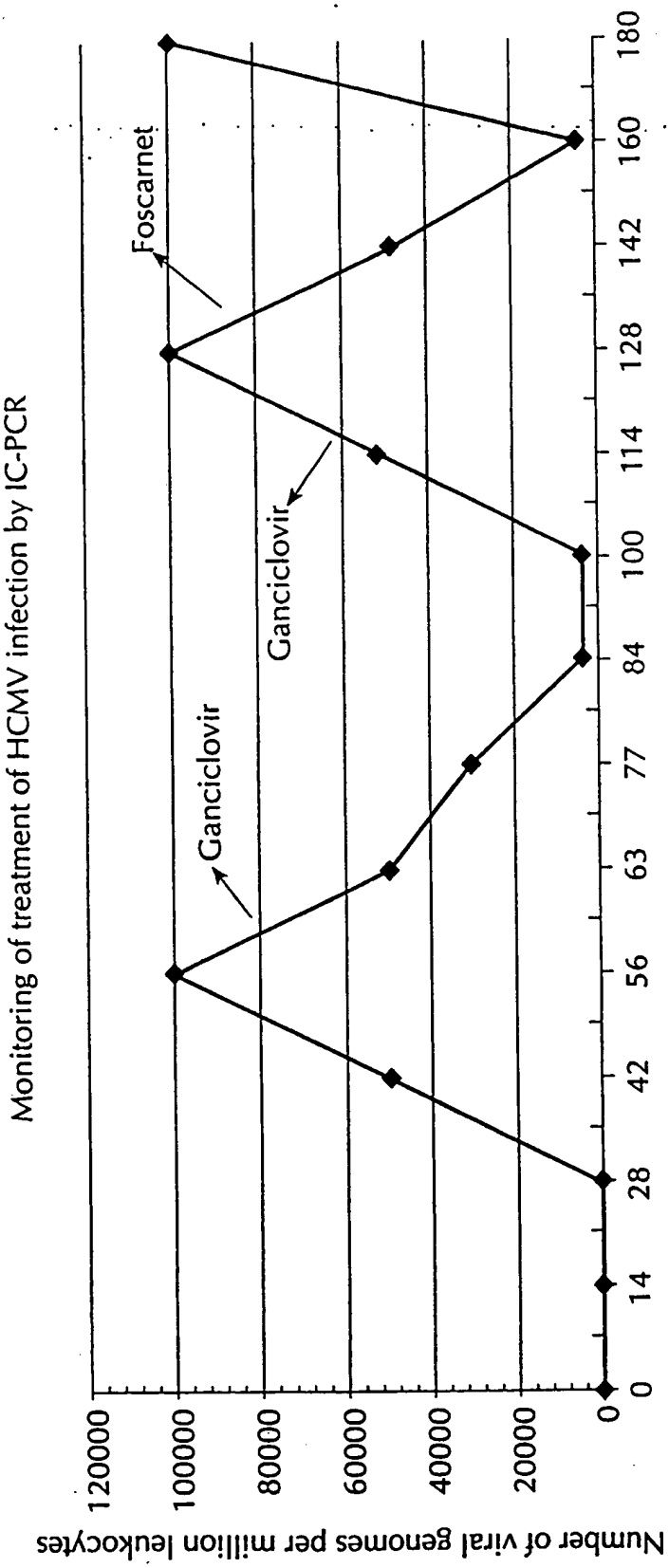
Monitoring of treatment of HCMV infection by IC-PCR
Number of viral genomes per million leukocytes
120000
100000
80000
60000
40000
20000
0
0
14
28
42
56
63
77
84
100
114
128
142
160
180
Days after transplant
Foscarnet
Ganciclovir
Ganciclovir

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16361

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C07H 21/02, 21/04
US CL :435/6; 536/23.1, 24.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,219,727 A (WANG et al) 15 June 1993, see the entire document.	1-3, 12, 14-15 --- 1-17,20-27
Y,P	US 5,674,680 A (SAKSELA et al) 07 October 1997, see the entire document.	4-10, 17
Y	ISRAELI et al. Sensitive Nested Reverse Poymerase Chain Reaction Detection of Circulating Prostatic Tumor Cells: Comparison of Prostate-Specific Membrane Antigen and Prostate-specific Antigen-based Assays. Cancer Research. 15 December 1994, Vo.l 54, pages 6306-6310, see the entire document.	10-11, 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 SEPTEMBER 1998	Date of mailing of the international search report 20 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ETHAN WHISENANT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16361

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KONDO et al. Human Cytomegalovirus Latent Infection of Granulocyte Macrophage Progenitors. Proc. Natl. Acad. Sci., USA. December 1994, Vol. 91, pages 11879-11883, see the entire document.	17, 22, 27
Y	US 4,965,188 A (MULLIS et al) 23 October 1990, see the entire document.	16
Y	CABALLERO et al. Low Stringency-PCR (LS-PCR) Allows Entirely Internally Standardized DNA Quantitation. Nucleic Acids Research. 1995, Vol. 23, No. 1, pages 192-193, see the entire document.	17
Y	PIATAK et al. High Levels of HIV-1 in Plasma During all Stages of Infection Determined by Competitive PCR. Science. 19 March 1993, Vol 259, pages 1749-1754, see the entire document.	20-27

INTERNATIONAL SEARCH REPORT

Intemational application No.
PCT/US98/16361

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Medline, WPIDS, USPATFULL

Quantitation, Gene, and Expression